INCUBATING THE NITROCELLULOSE FILTER WITH THE SECONDARY IMMUNOLOGICAL REAGENT

The secondary reagent (usually an anti-immunoglobulin or protein A) may be radiolabeled with ^{125}I as described on pages 18.24–18.25 or may be covalently coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. Covalently coupled immunoglobulin and protein A are sold commercially.

Although both radiolabeled and enzyme-coupled secondary reagents can work very well, antibodies are sometimes inactivated if the radiolabeling process is carried out too enthusiastically. Most workers therefore prefer to use enzyme-coupled reagents that have been tested by their commercial manufacturers.

Radiolabeled secondary reagents

1. Transfer the nitrocellulose filter from the final wash in phosphate-buffered saline (PBS; see Appendix B) (step 3, page 18.71) to a heat-sealable plastic bag (e.g., Sears Seal-A-Meal) containing 0.1 ml of fresh blocking solution per square centimeter of filter and add approximately 10^4 cpm of the radiolabeled secondary reagent per square centimeter of filter.

Blocking solution

5% (w/v) nonfat dried milk
0.01% antifoam A
0.02% sodium azide in PBS

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

If the background of nonspecific binding of immunological probes is unacceptably high, try adding Tween 20 to a final concentration of 0.02%. In most cases, the presence of this detergent will not affect specific binding of antibody to the target antigen.

2. Incubate the filter for 1–2 hours at room temperature with gentle agitation on a platform shaker.

3. Cut open the bag, and quickly transfer the filter to a tray containing 250 ml of PBS. Discard the plastic bag and radioactive fluid in the radioactive waste.

4. Wash the filter in several changes of PBS (10 minutes each change). Continue washing until no radioactivity is detected by a hand-held minimonitor in regions of the filter that carry no protein.

5. Remove the filter from the last wash, and allow it to dry for 10 minutes on a stack of paper towels.

6. Mount the filter on a piece of Whatman 3MM paper, and attach radioactive markers to the paper.

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Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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